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# Antioxidant properties of citrus fibre and the prediction of oxidation in ground beef meatballs made with citrus fibre by ATR-FTIR spectroscopy with principal component analysis

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**Abstract**

The objectives of the present work were (1) to determine the total polyphenol content (TPC), total flavonoid content (TFC), and oxygen radical absorbance capacity (ORAC) of citrus fibre, and (2) to predict, by attenuated total reflection (ATR)-Fourier transform infrared spectroscopy (FTIR), the oxidative stability of ground beef made with different levels (*i.e.,* 0, 1, 3, and 5%) of citrus fibre (CF) during 1, 3, 5, or 7 days of refrigerated storage. The TPC, TFC, and ORAC values of citrus fibre were 3.753 ± 0.49 mg/g, 2.825 ± 0.008 mg/g, and 10.036 ± 1.94 µmol/g in dry basis, respectively. The citrus fibre pH was 4.45 ± 0.075. To monitor lipid oxidation, the peaks at 2924, 2853, and 1743 $cm^{-1}$ were useful. Principal component analysis (PCA) was applied at a 5% significance level between 1780 - 1700 $cm^{-1}$. The results of the present work demonstrated that the addition of citrus fibre at higher levels (*i.e.,* 3 and 5%) had a pro-oxidative effect on ground beef meatballs. FTIR helped to predict oxidation in meat products.



## Introduction

Citrus is one of the leading types of fruits produced globally. The majority of citrus fruits are used for juice production; but, they are also used in the canning industry, and in essential oil production (Marín *et al.,* 2007). A large amount of waste is generated during juice production. This waste product is rich in fibre, phenolic compounds, and antioxidants (Gorinstein *et al.,* 2001; Fernández-López *et al.,* 2004; Tripoli *et al.,* 2007; Valdivia-Lopez, 2017). Therefore, antioxidant-rich fibre obtained from the citrus industry has great potential to be incorporated as a functional ingredient in comminuted meat products. It has been found that citrus fibre has great water- and oil-holding capacities (Lario *et al.,* 2004; Berk, 2016; Gedikoğlu and Clarke, 2019). Also, due to the presence of antioxidants, citrus fibre has been shown (by using the thiobarbituric acid reactive substances [TBARS] assay) to delay lipid oxidation in meat products such as bologna sausage (Fernández-Ginés *et al.,* 2003), Swedish meatballs (Fernández-López *et al.,* 2005), dry-cured sausages (Fernández-López *et al.,* 2007), minced fish (Sánchez-Alonso *et al.,* 2007), and chicken hamburgers (Sáyago-Ayerdi *et al.,* 2009). While the TBARS assay is a common protocol for determining lipid oxidation, it has some limitations. The use of simple, rapid, and precise methods such as Fourier transform infrared spectroscopy (FTIR) can effectively evaluate the lipid oxidation of fats and oils (Shahidi and Zhong, 2005). Infrared radiation is absorbed by the sample, some of which is transmitted, giving the molecular fingerprint of the sample. FTIR provides fast and accurate information about food samples, and it has been employed in the identification of oils such as sunflower oil (Guillén *et al.,* 2005), olive oil (Rohman *et al.,* 2014), and virgin coconut oil (Rohman, 2017). Additionally, FTIR has been used to determine the oxidative stability of ten edible oils (Guillén and Cabo, 2000), and the thermal stability of vegetable oils (Rohman and Che Man, 2013). Furthermore, FTIR has also been used to detect the adulteration of pure ghee with goat body fat (Upadhyay *et al.,* 2016), to authenticate halal meat (Rahmania *et al.,* 2015; Rohman *et al.,* 2016; Rahayu *et al.,* 2018), and wild sea bass (Vidal *et al.,* 2014). Similarly, Grunert *et al.* (2016) reported the use of FTIR to differentiate between fresh, frozen, and thawed chicken.

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Although FTIR has been employed in a variety of studies to identify and authenticate oils and fats, there are only a few studies investigating the oxidative stability of animal products. Using FTIR, Guillén and Cabo (2004) researched the oxidative stability of smoked pork in comparison to a control, while Guillén *et al.* (2004) used FTIR to determine the oxidative stability of salted and unsalted salmon. The present work is unique because it uses waste from the industry as the source of citrus fibre, which has not been previously studied. Also, thus far, there is no study related to the use of FTIR to predict the lipid oxidation over a shelf life of ground beef combined with an ingredient with antioxidant potential.

Therefore, the objectives of the present work were (1) to determine the antioxidant potential of a specific citrus fibre, and (2) to predict the lipid oxidation in ground beef meatballs made with 0, 1, 3, and 5% citrus fibre by employing attenuated total reflectance (ATR)-Fourier transform infrared spectroscopy (FTIR) coupled with chemometric analysis during seven days of refrigerated storage.

## Materials and methods

*Materials*

Folin-Ciocalteu phenol reagent was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Aluminium chloride anhydrous ($AlCl_3$), potassium acetate ($CH_3COOK$), and sodium carbonate ($Na_2CO_3$) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Quercetin and β-carotenoid were purchased from Sigma-Aldrich® Co. (St. Louis, MO, USA). Gallic acid was purchased from Fluka® Analytical (St. Louis, MO, USA). An ORAC antioxidant assay kit was purchased from Zen-Bio, Inc. (Research Triangle Park, NC, USA). Citrus fibre (CitraFiber™), which is a fine powder, was provided by the Natural Citrus Products Corporation (Fort Pierce, FL, USA).

*Sample preparation*

Sample preparation was performed following a method previously described by Gedikoğlu and Clarke (2019). After the meat samples were ground, they were separated into four treatment groups, and prepared according to their citrus fibre levels (*i.e.,* 0, 1, 3, and 5%) using CitraFiber™. This procedure was replicated twice to a total of three replicates.

*Extraction*

The extraction was performed following a method previously described by Sun *et al.* (2010). First, 10 g of citrus fibre was mixed with 200 mL of methanol (1:20, w/v). After sonicating the mixture for 1 h, the slurry was filtered using a Buchner funnel, vacuum flask, and Whatman® No. 1 filter paper; and then refrigerated in the dark. The solid collected from the filtration was put through the same procedure for two more times, until a faint yellow colour was observed in the filtrate. All the collected liquids were combined. Using a round-bottom flask and rotary evaporator, the methanol in the sample was completely evaporated at 50°C, and the residue was dissolved in 10 mL of methanol. This extract was used for the TPC, TFC, and ORAC assays.

*Determination of the total polyphenol content*

To determine the TPC, a procedure from Lin and Tang (2007) was adopted. Gallic acid was chosen as the standard, and a six-point standard curve was prepared (0 - 60 mg/L). The total polyphenol content of the citrus fibre was expressed as mg of gallic acid equivalents (GAE)/g for dry powder.

*Determination of the total flavonoid content*

To determine the TFC, a procedure from Lin and Tang (2007) was adopted. Quercetin was chosen as the standard, and a seven-point standard curve was prepared (0 - 100 mg/L). The total flavonoid content of the citrus fibre was expressed as mg of quercetin equivalents (QUE)/g for dry powder.

*Oxygen radical absorbance capacity assay*

A Zen-Bio kit (Zen-Bio, Inc., Research Triangle Park, NC, USA) was used for the ORAC assay. The Synergy™ HT 96-well microplate reader was used, the incubation chamber was set to 37°C (BioTek® Instruments, Inc., Winooski, VT, USA), the excitation wavelength was set to 485 nm, and the emission wavelength was set to 530 nm. A five-point (0 - 50 μM) standard curve was prepared using Trolox as the standard. The ORAC values of the samples were calculated based on Dávalos *et al.* (2004) and were expressed as μmol Trolox/g for dry powder.

*Citrus fibre pH*

Sample (10 g) was homogenised with 90 mL of distilled water using a blender. Then, the pH of the slurry was determined using a Fisher Accumet® model 230A pH/ion meter (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The pH measurements of the samples of the three replicates were determined in duplicates.

*Extraction of lipids*

To extract lipids from the ground shank

muscle, a procedure from Bligh and Dyer (1959) was adopted. The chloroform layer, which had the extracted fat, was analysed using ATR-FTIR.

*Determination of moisture and fat content*

The moisture and fat contents of the meat samples were determined based on the CEM® SMART Trac™ System (CEM Corporation, NC, USA). This two-step system first uses microwaves to determine the moisture content of a meat sample. First, samples weighing between 3 and 5 g were spread evenly across a tared square pad. The pad was covered with another tared square pad to make a sandwich. Then, the moisture content of the sample was measured. Later, low-resolution time domain nuclear magnetic resonance (LR-NMR) (CEM Corporation, NC, USA) was used to determine the fat content of the microwaved sample. The parameters used for LR-NMR analysis were: NMR-RF pulse generator, pulse power of 250 W nominal; pulse times, variable in 100 ns increments; transmit and receive phases, selectable 0, 90, 180, and 270°; and nominal 90° pulse times, 4 ms (18 mm probe). Meanwhile for the magnet: permanent, thermally stabilised, 0.47 T (20 MHz), and homogeneity better than 10 ppm. For the signal detection: dual-channel (quadrature) detection with programmable low-pass filtering, programmable data acquisition rate up to 4 MHz per pair of points (CEM Corp.), or equivalent. The sample sandwiched in the pads was rolled in Trac film. Then, the rolled sample was compressed into the plastic sleeve, and it was inserted into the NMR chamber for fat analysis (Keeton *et al.,* 2003).

*ATR-FTIR measurements*

For each of the three meat replicates, four solvent-extracted fat samples were collected for each treatment and each day. Each of the solvent-extracted fat samples was measured between 4000 and 600 cm $^{-1}$, at a resolution of 2 cm$^{-1}$, with a 128 interferogram scan using a Nicolet 380 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with Smart Orbit, which included a diamond plate (30000 - 200 cm$^{-1}$) and a variable pressure swivel tower. The spectra of each sample were corrected against the background spectrum of air to present the spectra in absorbance units. After each sampling, the surface of the diamond and the pressure swivel tower attachment were cleaned using 70% ethyl alcohol with Kimwipes™. A total of 12 measurements were daily collected for each treatment. DeLight software (D-Squared Development Inc., La Grande, OR, USA) was used to analyse the FTIR data. A trend analysis with fixed block mean, polynomial subtract (first-order), and smoothing (8 cm$^{-1}$) options was chosen to analyse all the data. Then, the averages of these 12 measurements for each daily treatment were used to obtain spectra figures.

*Principal component analysis*

To conduct PCA, FTIR-treated spectra between 1780 and 1700 cm$^{-1}$ were used. In this range, the development of a new peak was observed, so data from this range was used for the analysis. Data was normalised and scaled between [-1, 1]. The PCA was applied to the normalised data. FTIR data between 1780 and 1700 cm$^{-1}$ was reduced to three principal components. At this level of reduction, 95% of the data was covered. Orange (Demsar *et al.,* 2013) was the PCA tool, and MATLAB (MathWorks, 2019) was the graphic tool.

*Statistical analysis*

Three replications using citrus fibre were evaluated for their TPC, TFC, and ORAC values. Data were evaluated by analysis of variance (ANOVA), using the general linear model (GLM) procedure of the SAS Institute (SAS, 2011). Three replications of the ground beef meatballs were evaluated for moisture and fat contents. The ANOVA, using the GLM (SAS, 2011) procedure, was a randomised complete block in which the block was a carcass. The treatments were arranged as a 4 × 4 factorial (4 levels of citrus fibre, 4 different days). The means were separated by a Tukey's test when significant ($p < 0.05$) treatment effects were found.

## Results and discussion

*Total polyphenol, flavonoid, and carotenoid contents, and ORAC values*

Table 1 shows the total polyphenol content (TPC), total flavonoid content (TFC), oxygen radical absorbance capacity (ORAC) values, and the pH of the citrus fibre. For TPC, CitraFiber™ yielded 3.753 ± 0.49 mg/g db. Previous studies reported that phenolic compounds, particularly the flavonoids found, in citrus peel exhibited antioxidant properties (Morel *et al.,* 1993; Gorinstein *et al.,* 2001; Garau *et al.,* 2007). Oboh and Ademoson (2006) reported that the TPC of Nigerian orange peel was 3.2 mg/g db, and tangerine peel was 2.9 mg/g db. Castro-Vazquez *et al.* (2016) investigated the presence of total polyphenols, flavonoids, and the antioxidant capacity of grapefruit peel. Their study showed that the total polyphenols, flavonoids, and antioxidant capacity of dried (*i.e.,* 45 and 60°C) peels were higher than those of fresh peels. On the other hand, Rahman *et al.*

Table 1. Total polyphenol content, total flavonoid content, oxygen radical absorbance capacity, and pH of CitraFiber™.

| CitraFiber™ | |
|---|---|
| TPC (mg GAE/g) | 3.75 ± 0.49 |
| TFC (mg QUEE/g) | 2.83 ± 0.01 |
| ORAC value (μmol Trolox/g) | 10.04 ± 1.94 |
| pH | 4.45 ± 0.08 |

All measurements are the mean value ± SD of triplicate determinations.

(2018) found that albedo, flavedo, and lamella had higher TPC when fresh than dry. Chen *et al.* (2011) investigated the effects of different drying temperatures (*i.e.,* 50, 60, 70, 80, 90, and 100°C) on the phenolic content, flavonoid content, and antioxidant properties of sweet orange peel. They reported that drying at higher temperatures (70, 80, 90, and 100°C) resulted in higher flavonoid and phenolic contents, while antioxidant activity decreased significantly ($P < 0.05$) with an increase in the drying temperature to 100°C. Rahman *et al.* (2018) also found that while fresh pomelo by-products had a higher TPC, dry pomelo by-products had a higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. Rahman *et al.* (2018) suggested that due to a significant negative correlation between the TPC values and the DPPH radical scavenging activity, the DPPH radical scavenging activity was not affected by the presence of phenolic compounds. Jeong *et al.* (2004) suggested that exposure to heat during drying may lead to the development of low molecular weight phenolics that can contribute to higher antioxidant capacity. It was also suggested that not all phenolic compounds have good free scavenging activity (Jang *et al.,* 2010). Furthermore, it was reported that flavonoids with hydroxyl groups could be used as proton donors and can display radical scavenging activity (Chang and Azrina, 2017).

The CitraFiber™ had a TFC of 2.825 ± 0.008 mg/g db. Gorinstein *et al.* (2001) found that the TFC of the peel of oranges, lemons, and grapefruits was significantly higher ($p < 0.05$) than the TPC. Li *et al.* (2012) investigated varying storage conditions on TPC, vitamin C content, and the antioxidant potential of honey pomelo (*Citrus grandis*). They obtained a higher TPC, vitamin C content, and antioxidant potential for low-oxygen and passive atmospheric conditions than for high-atmospheric conditions. Comparing their results to our previous study (Gedikoğlu *et al.,* 2018), it showed that the TPC, TFC, and ORAC values were higher for citrus fibre obtained through regular washing in comparison to hot washing.

The ORAC assay determines the radical scavenging activity of tested samples towards peroxyl radicals generated during the thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). According to the United States Department of Agriculture database (USDA, 2010), the ORAC values of raw oranges of different varieties ranged from 9.84 - 21.03 μmol/g, while CitraFiber™ had an ORAC value of 10.036 ± 1.94 μ mol/g. Zhang *et al.* (2014) reported that wild Chinese mandarins had very high ORAC values of 395.66 - 834.37 μmol TE/g db. Park *et al.* (2014) determined the antioxidant potential of orange flesh and peel using different solvents for extraction, and recommended that acetone was the best solvent for the extraction of antioxidant compounds. We found similar results—that citrus fibre extracted with acetone yielded a higher TPC, TFC, and ORAC values in comparison to methanol extract (Gedikoğlu, 2015).

CitraFiber™ had a pH of 4.45. Lario *et al.* (2004) tested the pH of dried lemon fibre, and found it to be 3.83. The concentration and antioxidant activity of the phytochemicals found in citrus fruit differ based on where and how the citrus fruit is grown, the type and part of the citrus fruit used, and the processing conditions (Manthey and Grohmann, 1996; Gorinstein *et al.,* 2001; Nogata *et al.,* 2006; Gedikoğlu *et al.,* 2018; Rahman *et al.,* 2018). Previous studies evaluated the TPC, TFC, and ORAC values from fresh fruits as well as by-products obtained in a lab-scale experiment, whereas in the present work, these values measured CitraFiber™, a by-product of the fruit juice industry. The CitraFiber ™ used in the present work had TPC, TFC, and ORAC values within the range of the values found by earlier studies.

*Moisture and fat contents*

The results revealed that there were significant differences ($p < 0.05$) in moisture between the control and both CF 3% and CF 5%. The control had the highest moisture content (73.17 ± 0.99), followed by CF 1% (71.97 ± 0.98), CF 3% (70.42 ± 1.26), and CF 5% (68.88 ± 1.29). Also, there was no significant difference ($p > 0.05$) between the treatments for fat content, with the control having the highest fat content (4.7 ± 1.22%), followed by CF 1% (4.64 ± 1.27), CF 3% (4.31 ± 1.77), and CF 5% with the lowest fat content (4.18 ± 1.72).

*Results of the FTIR analysis*

The band assignments were achieved according to Guillén and Cabo (1997). The medium band at 3010 $cm^{-1}$ is associated with a cis-double bond stretching vibration. The very strong bands at 2924 and 2853 $cm^{-1}$ are associated, respectively, with the asymmetric and symmetric stretching of the aliphatic $CH_2$ functional group. The very strong band at 1743 $cm^{-1}$ is related to an ester carbonyl bond associated with triglycerides. The medium band at 1467 $cm^{-1}$ is related to the aliphatic stretching of the $CH_2$ and $CH_3$ functional groups. The medium bands at 1239 and 1164 $cm^{-1}$ are associated with ester stretching and $CH_2$ bending vibrations. The medium band at 1097 $cm^{-1}$ is associated with ester stretching, while the medium band at 723 $cm^{-1}$ is associated with a $CH_2$ rocking vibration and cis-disubstituted olefins.

The FTIR spectra of the beef fat with added fibre at four levels (*i.e.,* CF 0%, CF 1%, CF 3%, and CF 5%) displayed no difference in the peak appearance on day 1, but there were slight variations in absorbance between treatments due to difference in fat contents. To investigate the stages of oxidation, as well as the impact of different treatment levels on the oxidation, the peaks at 2924, 2853, and 1743 $cm^{-1}$ were investigated further.

Figure 1 presents the FTIR spectra of all the treatment levels between 2980 and 2880 $cm^{-1}$. Every decrease in absorbance showed the same pattern in all treatments. First, from day 1 to 3, the absorbance decrease was due to the production of peroxides. From day 3 to 5, the absorbance increase resulted from the production of secondary oxidation products. From day 5 to 7, the absorbance started to decrease due to the breakdown of secondary oxidation products. Also, Figure 1 illustrates that all the treatments had the highest absorbance at day 1, which decreased over time. While the change in the stages of oxidation was the same in all treatments, the drop in absorbance from day 1 to 3 was due to possible peroxide formation, which occurred much less in the control than in all the other treatments. The changes in absorbance were more apparent in CF 3% and CF 5%. Vlachos *et al.* (2006) tested the impact of heating on the oxidation of corn oil spectra. For the band at 2925 $cm^{-1}$, they found that with heating, the absorbance decreased, and the band width increased. They also tested the addition of oregano to the corn oil, and compared it with a control consisting of corn oil without oregano. The results demonstrated that oregano had antioxidant properties, and prevented a reduction in the valley.

Figure 2 displays the FTIR spectra of all the treatment levels between 2870 and 2820 $cm^{-1}$. Similar to Figure 1, the control treatment (*i.e.,* CF 0%) evidenced much less change in absorbance than each of the other treatments, and change was more apparent in CF 3% and CF 5%. Guillén *et al.* (2004) investigated the oxidative stability of salted and unsalted salmon. They reported that dry salting caused salmon to oxidise. Guillén and Cabo (2004) investigated the impact of smoke flavourings on the oxidative stability of pork adipose tissue, finding that liquid smoke flavouring improved oxidative stability.

Figure 3 presents the FTIR spectra of all the treatments between 1780 and 1700 $cm^{-1}$. The results

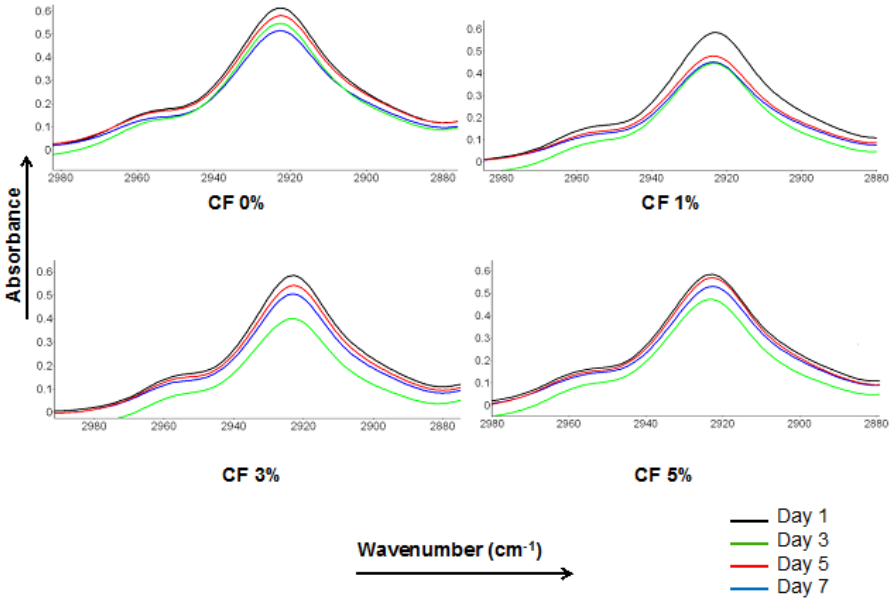


Figure 1. FTIR spectra of beef fat from ground shank muscle, with and without citrus fibre, between 2980 and 2880 $cm^{-1}$.

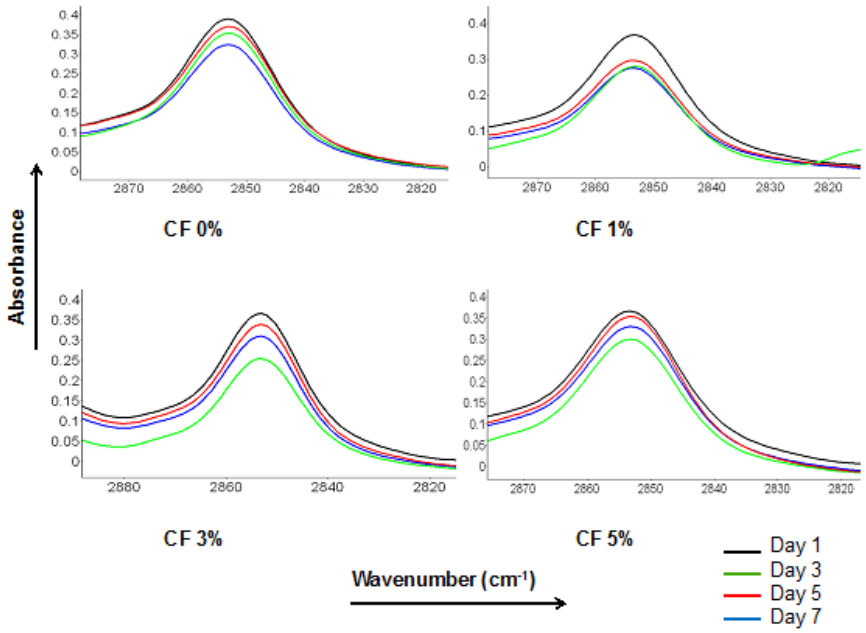


Figure 2. FTIR spectra of beef fat from ground shank muscle, with and without citrus fibre, between 2870 and 2820 $cm^{-1}$.

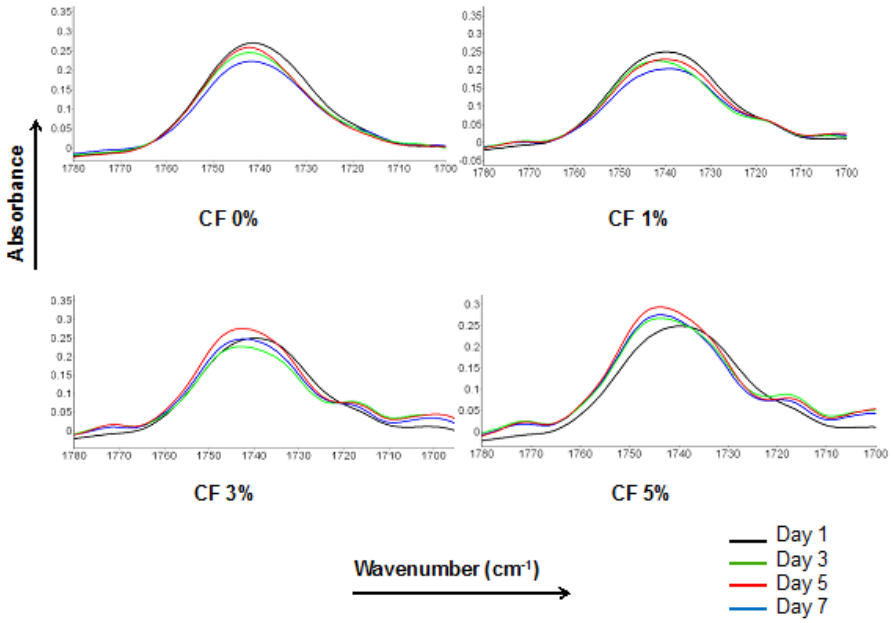


Figure 3. FTIR spectra of beef fat from ground shank muscle, with and without citrus fibre, between 1780 and 1700 $cm^{-1}$.

revealed that there was a slight widening in the band from day 1 to 7 for all the treatments. Furthermore, the spectra of CF 1%, CF 3%, and CF 5% started to develop peak areas from 1720 - 1710 $cm^{-1}$ by day 3. This peak was more apparent for CF 3% and CF 5%. Earlier studies have reported that there was absorbance of aldehydes and ketones at around 1728 $cm^{-1}$ (Guillén and Cabo, 2004; Guillén *et al.,* 2005). It could be that this weak band appears due to the oxidation of lipids and the production of aldehydes and ketones. Rohman and Che Man (2013) also observed the increase in a peak between 1744 and 1655 $cm^{-1}$ due to the formation of carbonylic compounds during the thermal oxidation of vegetable oils. Although it has been reported that citrus fruits and their peels are rich in antioxidants due to vitamins, flavonoids, and phenolic compounds (Zou *et al.,* 2016), some of these natural antioxidants can have a pro-oxidative effect, depending on their concentrations. Lauritzen and Olsen (2004) found that when less than 50 ppm of antioxidant ascorbic acid was added in combination with 5 ppm copper, it delayed lipid oxidation in chemical oxygen demand (COD). However, when it was administered at 500

ppm without copper, it had a pro-oxidative effect. Yalınkılıç *et al.* (2012) also reported that with an increase in citrus fibre levels (*i.e.,* 2 or 4%), the TBARS values of fermented sausages also rose.

*Results of the principal component analysis*

The cumulative *R*-squared for the PCA was 0.96. The variance explained by the first component was 0.857, 0.06 by the second component, and 0.043 by the last component. Figure 4 illustrates the linear projection of treatments at different storage days based on the principal components. Figure 4 shows that all the treatments (*i.e.,* control, CF 1%, CF 3%, and CF 5%) were located at the centre at day 0. At day 0, there was no oxidation. On the next storage day (day 3), both the control sample and CF 1% sample were located at the centre, while CF 3% and CF 5% moved away from the centre. This could indicate the start of oxidation for those treatments. On storage day 5, treatments with citrus fibre were not near the centre, while the control treatment was still at the centre. CF 1% started to separate from the centre, which signifies oxidation. On the final day of storage (day 7), all the treatments had accumulated together and away from the centre, indicating that all the treatments had oxidised. These results demonstrate that when citrus fibre is mixed into raw ground beef samples at various levels, it had an oxidative effect over time based on the level added.

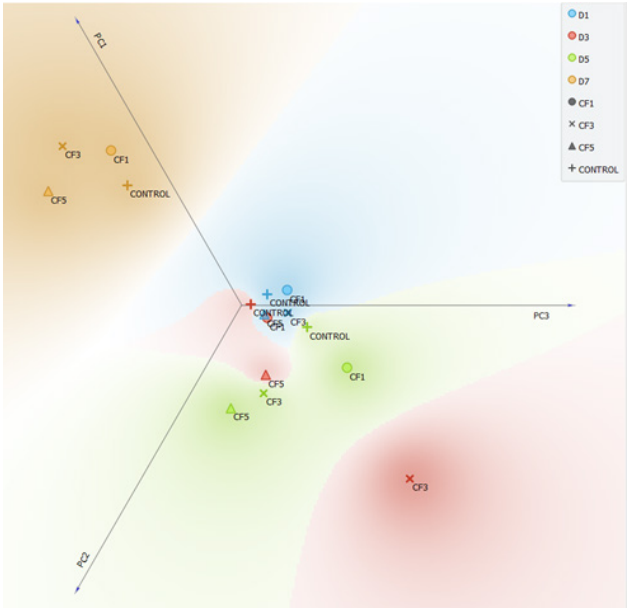


Figure 4. Principal component analysis (PCA) score plot of spectral data between 1780 and 1700 $cm^{-1}$ obtained from fresh ground beef, added with different levels of citrus fibre at different storage days (colour represents "day", shape represents "treatment").

## Conclusion

The objectives of the present work were to determine the antioxidant potential of citrus fibre, and to predict, using ATR-FTIR, the oxidative stability of ground beef meatballs prepared with different levels of citrus fibre. Results indicated that the TPC, TFC, TCC, and ORAC values of citrus fibre were within the range of those found in earlier studies. Citrus fibre at 3 or 5% levels in ground beef exerted pro-oxidative effect by qualitative analysis using FTIR data and PCA. Peaks at 2924, 2853, and 1743 $cm^{-1}$ were effective for monitoring lipid oxidation. Hence, citrus fibre should be applied at levels less than 3% in meat products due to a possible pre-oxidative effect. The present work is unique based on the use of citrus fibre obtained as a waste material of the juice industry, while other studies have used the processed peel of fresh citrus fruit. Also, thus far, it is the only study that employs FTIR coupled with PCA to predict lipid oxidation in a meat product over a shelf life.

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